Short communication

Evaluation of the protective effects of α-tocopherol and retinol against ochratoxin A cytotoxicity

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Ochratoxin A (OTA), a mycotoxin frequently present in food and feedstuffs, produces a wide range of toxic effects, including cell death via lipid peroxidation. In one human and four animal cell lines we determined the half lethal concentration (LC_{50}) of OTA, its effect on reactive oxygen species (ROS) production, and its ability to induce cytochrome p450 activity. We also examined the protective effect of α-tocopherol and all-*trans*-retinol in the most sensitive cell lines (i.e. bovine mammary epithelia, for which LC_{50} was 0·8 μg/ml (24 h), and Madin Darby canine kidney, for which LC_{50} was 4·3 μg/ml (48 h)). Pre-incubation for 3 h with either antioxidant significantly ($P < 0·05$) ameliorated the OTA-induced reduction in cell viability and significantly decreased ($P < 0·05$) ROS production. These findings indicate that oxidative stress is an important factor in OTA cytotoxicity. Supplementation with antioxidant molecules may counteract the short-term toxicity of this mycotoxin.

Ochratoxin A: Antioxidants: Cell cultures: Reactive oxygen species

Ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus* sp., *Penicillium verrucosum* and other moulds (Hussein & Brasel, 2001). Chemically it consists of a dihydroisocoumarin moiety linked via its carboxyl group to the amine of L-phenylalanine. OTA is a potent nephrotoxin and genotoxin and is easily transformed into mutagenic metabolites (Schwerdt *et al.* 1999), considered by the International Agency for Research on Cancer as possibly carcinogenic (group 2B) for man (International Agency for Research on Cancer, 1993), so that steady exposure to OTA must be a cause for serious concern. For chemical carcinogens, the time–dosage profile is more important than mere dose (Petzinger & Ziegler, 2000). OTA is a widespread contaminant of human foods and animal feeds. In Europe OTA is one of the most widespread mycotoxins (Joint FAO/WHO Expert Committee on Food Additives, 2001). The European Union has established a maximum level of 5 parts per billion for OTA in wheat, barley, rye and derived products.

OTA toxicity has been attributed to the open lactone moiety which is structurally analogous to mitochondrial enzyme active sites and competitively binds enzyme substrates (Hussein & Brasel, 2001). OTA is a competitive inhibitor of ATPase, succinate dehydrogenase, and cytochrome C oxidase in rat liver mitochondria. Schaaf *et al.* (2002) attributed proximal tubule cell damage caused by OTA to the formation of reactive oxygen species (ROS) ($O_2^{\cdot-}$, $^{\cdot}OH$, $ROO^{\cdot}$) which induced a wide range of lesions on membranes, proteins, and nucleic acids. de Groene *et al.* (1996) also found that cytochrome p450 stimulated OTA-dependent lipid peroxidation and transformed non-mutagenic OTA into mutagenic metabolites. In a reconstituted microsomal system containing NAPDH–cytochrome p450 reductase, EDTA, Fe ions and NADPH, OTA was found to stimulate lipid peroxidation. OTA chelates Fe^{3+}, forming an OTA–Fe^{3+} complex, which is readily reduced by NAPDH–cytochrome p450 reductase to an OTA–Fe^{2+} complex. The rate of reduction of Fe^{3+} to Fe^{2+} increases in

the presence of OTA (Omar *et al.* 1991); while in the presence of oxygen the OTA–Fe^{2+} complex provides the active species that initiate lipid peroxidation (Baudrimont *et al.* 1997).

Deficiencies of nutritional factors may enhance individual susceptibility to the toxic effects of mycotoxins (Atroshi *et al.* 2002). Several natural components of the diet, for example, α-tocopherol, carotenoids, and ascorbic acid, show antioxidant properties and may counteract mycotoxin cytotoxicity by blocking mycotoxin-induced free radical damage (Galvano *et al.* 2001). Grosse *et al.* (1997) found that pre-treatment with antioxidant vitamins E, A or C reduced levels of DNA adducts in the kidney of mice administered OTA.

In the present study, we investigated the inhibitory effects of α-tocopherol and all-*trans*-retinol on the cytotoxicity of OTA in cultured cell lines.

Materials and methods

Materials

OTA, α-tocopherol, and all-*trans*-retinol were purchased from Sigma (St Louis, MO, USA). OTA was dissolved in methanol to obtain a stock solution of 40 μg/ml. The stock solution was further diluted in serum-free culture medium (containing 0·2 % (v/v) methanol).

Appropriate concentrations of antioxidants were determined in pre-test experiments (Baldi *et al.* 2002; Cheli *et al.* 2003). In subdued lighting, racemic α-tocopherol and all-*trans*-retinol were dissolved in ethanol to give 10 mM solutions. Further dilution with serum-free culture medium produced concentrations of 10 μM and 1 nM. These diluted stock solutions were stored in the dark before addition to cultures housed in a cabinet without lighting.

Cell lines and cell culture

We employed five well-characterised human and animal cell lines: human neuroblastoma (SK-N-MC), Madin Darby canine kidney (MDCK), mouse liver hepatocytes-12 (AML-12), pig kidney (LLC-PK1) and bovine mammary epithelium (BME-UV1). The first four cell lines were obtained from the cell-culture collection of Istituto Zooprofilattico Sperimentale (Brescia, Italy); BME-UV1 was obtained from the University of Vermont and maintained in our laboratory. The cell lines used in the present study were derived from tissues sensitive to OTA; their sensitivity to OTA has been reported by several studies (Robbana-Barnat *et al.* 1989; Bruinink & Sidler, 1997; Gekle *et al.* 2000). We also verified that these were sensitive in pre-experimental tests (Baldi *et al.* 2002).

Cells were cultured in sterile plastic flasks coated with a thin layer of complete culture medium (Sigma, St Louis, MO, USA) specific for the cell line, supplemented with 10 % (v/v) fetal calf serum, penicillin (10^5 IU/l) and streptomycin (100 mg/l). At confluence cells were detached from the flasks by trypsinisation and re-suspended in culture medium to a concentration of $2·5 \times 10^5$ cells/ml. Portions (200 μl) of cell suspension were dispensed into sixty wells of a ninety-six-well tissue culture plate. A confluent layer of cells formed after 24 h of incubation at 37°C in an atmosphere of 5 % (v/v) CO_2.

Determination of half lethal concentration of ochratoxin A

Cell viability after incubation with OTA was determined by measuring the production of the chromophore formazan from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) (St Louis, MO, USA). Formazan is produced in viable cells by the mitochondrial enzyme succinate dehydrogenase.

In dose–response experiments, cells were exposed to increasing concentrations of OTA (0–40 μg/ml). After 24 and 48 h of incubation, 150 μl MTT stock solution (5 mg/ml) in PBS was added to each well and the plates were incubated for 3 h at 37°C in a humidified chamber. The reaction was terminated by removing the incubation solution and adding 150 μl dimethyl sulfoxide to dissolve the formazan. The optical density of the dimethyl sulfoxide solution at 540 nm was determined on a Biorad 680 microplate reader (Biorad, Veenendaal, The Netherlands). Cells incubated with culture medium alone, representing 100 % viability, were included as negative controls in all experiments. The percentage cytotoxicity was calculated as follows:

$$\text{Percentage cytotoxicity} = 1 - (\text{mean optical density in presence of OTA/mean optical density of negative control}) \times 100.$$

From these data the half lethal concentration (LC_{50}) of OTA for each cell line was calculated. At least three replicates at each incubation time were performed and all experiments were performed at least twice.

Cytochrome p450 activity

The activity of cytochrome p450 1A1 (Cyp 1A1) was determined as an indicator of the ability of the cell lines to detoxify drugs. This was assessed as ethoxyresorufin-*O*-deethylase activity towards the artificial mono-oxygenase substrate 7-ethoxyresorufin (Donato *et al.* 1993) after incubation with the inducer β-naphthoflavone (Sigma, St Louis, MO, USA). Cell monolayers in ninety-six-well plates were exposed to various concentrations (5–20 μM) of β-naphthoflavone for 24 h, and washed twice with PBS (20 mM, pH 7·2, containing 9 g NaCl/l) at 37°C to remove detached cells. Then 200 μl specific culture medium/well, as described by Donato *et al.* (1993), was added. The plates were incubated for 1 h at 37°C in a humidified atmosphere and the supernatant fraction centrifuged at 300 *g* for 5 min. A portion (175 μl) of centrifuged supernatant fraction of each sample was transferred to a tube and 50 μl hydrolysis solution (10·2 mg β-glucuronidase and 100 IU arylsulfatase dissolved in 0·1 M-sodium acetate buffer (10 ml), pH 4·5) was added. The tubes were incubated for 3–12 h at 37°C in a shaking water bath. After incubation, 100 μl methanol was added to each tube followed by centrifugation (300 *g*

for 5 min). The fluorescence of resorufin in the supernatant fraction was measured with a spectrofluorimeter at 530 and 585 nm. A standard curve was prepared from known concentrations of resorufin in culture medium. Standards were processed as described for samples.

Determination of reactive oxygen species

ROS production was measured by the conversion of non-fluorescent 2′-7′-dichlorofluorescin diacetate to fluorescent 2′-7′-dichlorofluorescin (Kanthasamy *et al.* 1997). In dose–response experiments, cells were suspended in Krebs–Ringer solution (Ca–Krebs–Ringer buffer: NaCl, 125 mM; KCl, 5 mM; HEPES, 25 mM; glucose, 6 mM; $NaHCO_3$, 5 mM; $MgSO_4$, 1·2 mM; $CaCl_2$, 1·2 mM; pH 7·4) to which was added 5 μM-2′-7′-dichlorofluorescin diacetate followed by incubation for 15 min at 37°C. Cells were then washed twice with Ca–Krebs–Ringer buffer and incubated for 3 h with 5, 10, or 20 μg OTA/ml in a saline buffer. The fluorescence was measured with a spectrofluorimeter at excitation wavelength 475 nm and emission wavelength 525 nm. ROS production was expressed as the fluorescence in the treated samples compared with the control cells cultured without OTA ((fluorescence treatment/fluorescence control) × 100).

Inhibitory effects of antioxidants

The cell lines most sensitive to OTA (BME-UV1 and MDCK) were cultured with LC_{50} doses of OTA toxin in the presence or absence of α-tocopherol and all-*trans*-retinol (10 μM and 1 nM). Cell viability and ROS production after antioxidant treatment were assessed as described earlier (pp. 508–509).

To determine cell viability, cells were first pre-incubated for 3 h with antioxidants and then exposed to increasing OTA concentrations for 24 and 48 h. Cells were also exposed to α-tocopherol and all-*trans*-retinol alone and to ethanol (solvent for α-tocopherol and retinol) in absence of OTA to assess any effect on cell viability. Inhibition of cytotoxicity was determined by the MTT test and calculated as the percentage inhibition (percentage cytotoxicity of OTA – percentage cytotoxicity of (OTA + antioxidant)).

To determine ROS production, the cell lines most sensitive to OTA (BME-UV1 and MDCK) were pre-incubated for 3 h with either α-tocopherol (10 mM, 10 μM or 1 nM) or retinol (10 mM, 10 μM or 1 nM) and then exposed to 5, 10, or 20 μg OTA/ml for 24 or 48 h. ROS production after antioxidant treatment was determined as described earlier (p. 509), and the results were expressed as percentage inhibition of ROS production compared with incubation without antioxidant.

Statistical analysis

The data are presented as means and standard deviations. Linear regression analysis was performed to correlate OTA concentrations with percentage of inhibition of cell viability. The effect of the various treatments was assessed by ANOVA (general linear models procedure of SAS Institute, Inc., 1985); Duncan's multiple-range test was used *post hoc*, with $P < 0.05$ considered significant.

Results

Cytotoxic effects of ochratoxin A

In Table 1 LC_{50} evaluated after 24 and 48 h OTA treatment are reported. BME-UV1 and MDCK were the most sensitive to OTA (LC_{50} of 0·8 μg/ml after 24 h and 4·3 μg/ml after 48 h, respectively) and AML-12 was the most resistant ($LC_{50} > 40$ μg/ml after 24 h and 26·5 μg/ml after 48 h).

Cytochrome p450 activity

For the BME-UV1 cell line, β-naphthoflavone caused no change in Cyp 1A1 activity compared with the control cultures without β-naphthoflavone. For the AML-12 and LLC-PK1 cell lines we found a dose-dependent increase in Cyp 1A1 activity with increasing β-naphthoflavone concentration.

Ochratoxin A-induced reactive oxygen species production

Fig. 1 shows data on ROS production by the cell lines in the presence of different concentrations of OTA for 24 and 48 h. For all cell types, ROS production increased with OTA dose at both incubation times. ROS production was significantly higher ($P < 0.05$) in BME-UV1, MDCK and LLC-PK1 than in AML-12 at 24 h. After 24 h, the ROS production was significantly ($P < 0.05$) higher in BME-UV1 than other cell lines at all OTA concentrations. ROS production was always low in AML-12 cells and did not change significantly with OTA dose.

Effect of antioxidants on ochratoxin A cytotoxicity

The inhibitory effect of antioxidant agents on OTA cytotoxicity was evaluated in two ways: the MTT test and ROS production. α-Tocopherol and all-*trans*-retinol significantly ($P < 0.05$) reduced the loss of OTA-induced cell viability as determined by the MTT test: α-tocopherol by 10 % at 1 nM over 24 h in BME-UV1 cells; retinol by 33 % at 10 μM over 48 h in MDCK cells. The concentrations of methanol and ethanol used had no effect on cell vitality.
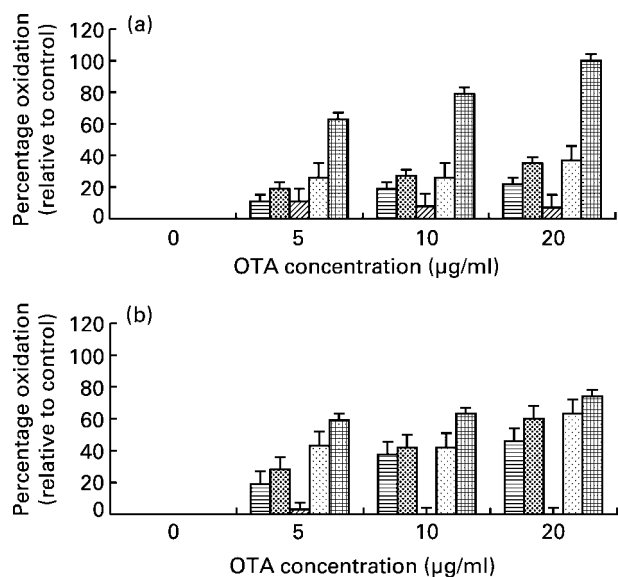
The effects of 3 h pre-incubation with retinol and α-tocopherol at concentrations of 10 mM, 10 μM and 1 nM on

Table 1. Toxicity of ochratoxin A (OTA) at 24 and 48 h on various cell lines, expressed as half lethal concentrations (LC_{50})

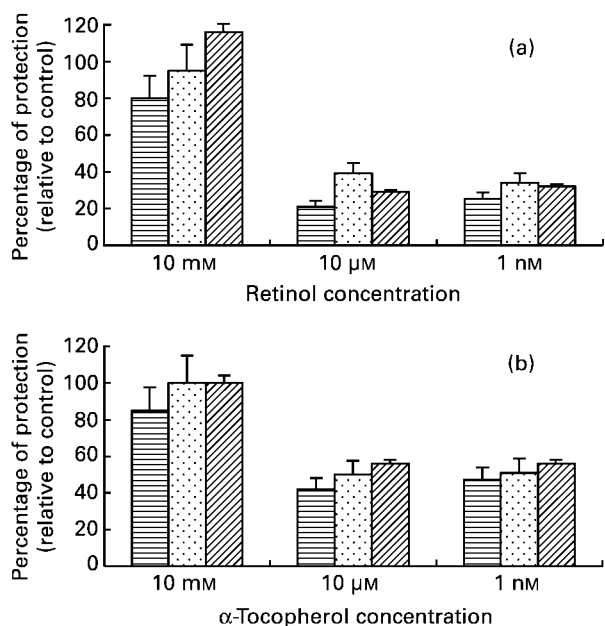| Cell lines	LC_{50} (μg/ml)	
	24 h	48 h
SK-N-MC (human neuroblastoma)	26·8	22·2
MDCK (Madin Darby canine kidney)	14·5	4·3
AML-12 (mouse liver hepatocytes-12)	>40	26·5
LLC-PK1 (pig kidney)	26·7	8·8
BME-UV1 (bovine mammary epithelium)	0·8	<DL*

< DL, below detectable limit.
* All OTA concentrations assayed on BME-UV1 cells led to 100 % loss of viability.
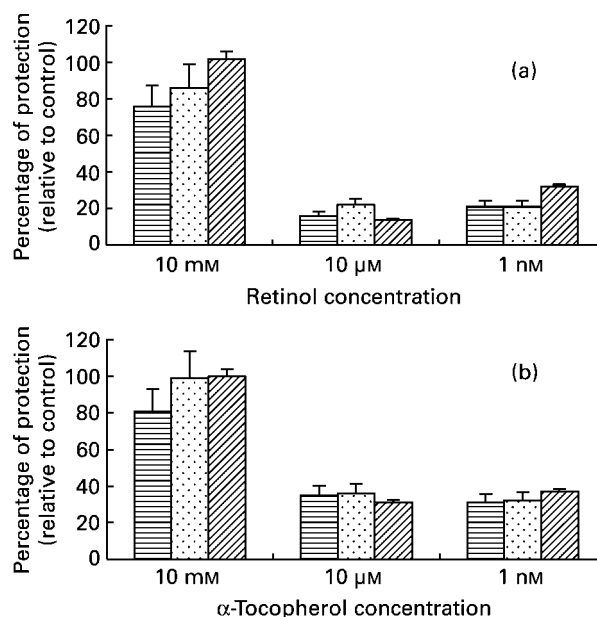
Fig. 1. Reactive oxygen species formation in the five cell lines after 24 h (a) and 48 h (b) of incubation with different concentrations of ochratoxin A (OTA). Values are means, with standard errors of the mean represented by vertical bars. (▤), SK-N-MC (human neuroblastoma cell line); (▨), MDCK (Madin Darby canine kidney cell line); (▧), AML-12 (mouse liver hepatocytes-12 cell line); (▩), LLC-PK1 (pig kidney cell line); (▦), BME-UV1 (bovine mammary epithelium cell line).

OTA-induced ROS production in the MDCK and BME-UV1 cell lines, respectively, are shown in Figs. 2 and 3 in the presence of OTA at 5, 10 and 20 μg/ml. These results are data from several pooled wells. At all concentrations of both antioxidants, the decrease in ROS

Fig. 2. Effect of retinol (a) and α-tocopherol (b) on ochratoxin A (OTA)-induced reactive oxygen species response in Madin Darby canine kidney (MDCK) cells. (▤), 20 μg OTA/ml; (▦), 10 μg OTA/ml; (▨), 5 μg OTA/ml. Values are means, with standard errors of the mean represented by vertical bars. For both antioxidants, mean values were significantly different ($P < 0.05$): 20 *v.* 10 μg OTA/ml; 10 mм *v.* 10 μм; 10 mм *v.* 1 nм.

Fig. 3. Effect of retinol (a) and α-tocopherol (b) on ochratoxin A (OTA)-induced reactive oxygen species response in bovine mammary epithelium (BME-UV1) cells. (▤), 20 μg OTA/ml; (▦), 10 μg OTA/ml; (▨), 5 μg OTA/ml. Values are means, with standard errors of the mean represented by vertical bars. For both antioxidants, mean values were significantly different ($P < 0.05$): 20 *v.* 10 μg OTA/ml; 10 mм *v.* 10 μм; 10 mм *v.* 1 nм.

production, compared with control incubations in the presence of the same concentration of OTA, was significant ($P < 0.05$). However, treatment with a high concentration (10 mм) of either antioxidant resulted in a significantly greater percentage protection than lower concentrations (10 μм and 1 nм).

Discussion

The present study has shown that the human neuroblastoma cell line SK-N-MC, and the animal cell lines MDCK, AML-12, LLC-PK1, and BME-UV1 have differing susceptibilities to OTA (Table 1). The most resistant was AML-12, derived from mouse liver hepatocytes, and the most sensitive was BME-UV1 derived from bovine mammary epithelium. MDCK, from dog kidney, was also highly sensitive to OTA, consistent with findings that kidney is a major target of the toxin *in vivo* (Grosse *et al.* 1997), although the LLC-PK1 cell line from pig kidney was somewhat less sensitive (Table 1). SK-N-MC, from human neuroblastoma, had limited sensitivity to OTA, although OTA neurotoxicity is well documented (Bruinink & Sidler, 1997; Bruinink *et al.* 1998). This is the only tumour cell line we tested, and we hypothesise that the ability of these cells to metabolically activate OTA is reduced.

Our findings on Cyp 1A1 activity are consistent with our sensitivity findings. In particular, OTA did not affect β-naphthoflavone-induced Cyp 1A1 activity in BME-UV1 cells, while in AML-12 cells, the most resistant, OTA caused a dose-dependent increase in Cyp 1A1 activity. Furthermore, in this cell line ROS production was lower than in the other cell lines and not dose dependent. These findings suggest that peroxidation is the

principal damage mechanism in BME-UV1 and in the other sensitive cell lines, while in AML-12 other mechanisms are involved.

Rodent mammary gland is a target of OTA toxicity (Hallén *et al.* 1998; Ferrufino-Guardia *et al.* 2000). Although in ruminants OTA is extensively degraded by ruminal protozoa and bacteria (Hult *et al.* 1976; Ribelin *et al.* 1978), if this system were saturated then mammary cells might be a target since the present data showed that bovine mammary epithelia were highly sensitive to OTA *in vitro*, as also indicated by ROS production. In fact, in all five cell lines, exposure to OTA increased ROS production in a concentration-dependent manner. This finding is further evidence that OTA cytotoxicity is principally due to oxidative damage, particularly since the cell lines that had the greatest sensitivity (low LC_{50}) to OTA were also characterised by high ROS production.

The main aim of the present study was to investigate the effects of vitamins A and E (as racemic α-tocopherol and all-*trans*-retinol) on OTA cytotoxicity in sensitive cell lines. We found that both retinol and α-tocopherol significantly inhibited OTA-induced ROS production in the most sensitive cell lines (BME-UV1 and MDCK) and this inhibition was concentration dependent. α-Tocopherol functions as a chain reaction-terminating antioxidant, by donating its phenolic hydrogen to a chain-propagating lipid peroxyl radical and producing the less reactive α-tocopheroxyl radical (Zhang & Omaye, 2001). The mechanism underlying the protective activity of all-*trans*-retinol in OTA treatment may be more complex, and is probably multifactorial. Vitamin A is known to protect epithelia by acting as a cross-linkage agent between lipid and protein, thereby stabilising the membrane (McDowell & Cunha, 1989). However retinol is also a strong inhibitor of xenobiotic oxidations catalysed by recombinant Cyp 1A1, cytochrome p450 2C8 and 2C19 (Yamazaki & Shimada, 1999). We found that all-*trans*-retinol exerted significant anti-toxic activity in OTA-treated cells and in particular caused a significant decrease in OTA-induced ROS production. We suggest that localisation of all-*trans*-retinol within the membrane might have played a crucial role in scavenging peroxyl radicals and stabilising the membrane (Tesoriere *et al.* 1996).

In conclusion, although it seems that OTA functions in sensitive cell lines by increasing ROS-induced cell damage, it is important to improve our knowledge of the mechanisms underlying OTA-induced cell damage, by investigating whether the compound forms DNA adducts and whether vitamin A and vitamin E together have synergistic effects against OTA toxicity.

Acknowledgements

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